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Mechanisms of Alkylpyrazine Formation in a Potato Model System Containing Added Glycine

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The use of glycine to limit acrylamide formation during the heating of a potato model system was also found to alter the relative proportions of alkylpyrazines. The addition of glycine increased the quantities of several alkylpyrazines, and labeling studies using [2-^{13}C]glycine showed that those alkylpyrazines which increased in the presence of glycine had at least one ^{13}C-labeled methyl substituent derived from glycine. The distribution of ^{13}C within the pyrazines suggested two pathways by which glycine, and other amino acids, participate in alkylpyrazine formation, and showed the relative contribution of each pathway. Alkylpyrazines that involve glycine in both formation pathways displayed the largest relative increases with glycine addition. The study provided an insight into the sensitivity of alkylpyrazine formation to the amino acid composition in a heated food and demonstrated the importance of those amino acids that are able to contribute an alkyl substituent. This may aid in estimating the impact of amino acid addition on pyrazine formation, when amino acids are added to foods for acrylamide mitigation.

KEYWORDS: Pyrazines; glycine; acrylamide; potato; dihydropyrazines; α-dicarbonyls; Strecker aldehydes; 2,3-butanedione; formaldehyde

INTRODUCTION

Addition of glycine has been proposed as a means to reduce acrylamide levels in carbohydrate-rich heated foods (*1*, *2*), but this may have a significant impact on the flavor profile of the final product. Alkylpyrazines have been reported to be important flavor volatiles in baked potato (*3*, *4*). However, recent work in this laboratory showed that glycine significantly altered the alkylpyrazine distribution pattern, and to a lesser extent, the total alkylpyrazine yield, of a potato model system cooked at 180 °C for 15−60 min (*5*). The increase in overall alkylpyrazine yield resulted from significant increases in the formation of certain key alkylpyrazines, notably those with more than one alkyl substituent, including a methyl or ethyl group in the 2- and 3-position on the pyrazine ring. These increases were offset by decreases in other alkylpyrazines. Such changes in the alkylpyrazine distribution pattern would be expected to alter the flavor properties of the final product, particularly since the yield of many alkylpyrazines with low odor thresholds (e.g., those with ethyl substituents) was diminished with glycine addition. Therefore, there is a need to understand how amino acid additives, like glycine, could affect flavor volatile formation in heated foods when used for acrylamide mitigation.

We proposed that the increase in 2,3-dimethyl substituted alkylpyrazines with glycine may be due to the observed increase in 2,3-butanedione (*5*). This α-dicarbonyl can be converted to 2-amino-3-butanone via a Strecker degradation reaction with an α-amino acid, providing the necessary α-aminocarbonyl precursor for the formation of these 2,3-dimethyl substituted pyrazines. Moreover, Yaylayan and Keyhani (*6*) found that 70% of the 2,3-butanedione generated in a [2-^{13}C]glycine−glucose model system was singly labeled, indicating that a glycine subunit had been incorporated into the 2,3-butanedione formed. However, the observed increase in two of the 2-ethyl-3-methyl substituted pyrazines could not be explained by an analogous increase in 2,3-pentanedione, which would yield 3(2)-amino-2(3)-pentanone through Strecker degradation. In fact, 2,3-pentanedione formation decreased with added glycine, suggesting that glycine incorporation into the 2-ethyl-3-methyl substituted pyrazines was via a different pathway (*5*). Therefore, the main aim of the work described here is to understand better the effect of glycine addition on alkylpyrazine formation mechanisms through labeling studies with [2-^{13}C]glycine.

Many studies on alkylpyrazine formation have been conducted with simple sugar−amino acid or sugar−ammonia model systems. These have employed various solvents, including deodorized cocoa butter−water, to study the effect of lipids on pyrazine formation (*7*). However, most of these studies have emphasized the reaction between a single amino acid and a sugar, as highlighted by Hwang et al. (*8*), who investigated the interaction of two different amino acids and one sugar in the same reaction medium by observing the pyrazine yields of different mixtures of glucose, ^{15}N-glycine, and a chosen amino acid. In this way, they were able to compare the contributions

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of ^{15}N-labeled glycine and the chosen amino acid to pyrazine formation and obtain some information on the competition between glycine and other amino acids in volatile formation. Nevertheless, these experiments were still very much limited to sugar−amino acid model systems. No labeling studies on alkylpyrazines have been reported using a real food matrix with an inherent sugar and amino acid content. A further objective of this present study is to provide greater insight into competing alkylpyrazine formation pathways in a typical food matrix represented by the potato model system.

MATERIALS AND METHODS

Chemicals. Unlabeled glycine (99+%) from the Sigma-Aldrich Company Ltd (Gillingham, U.K.) and [2-^{13}C]glycine (99%) from Cambridge Isotope Laboratories (Andover, MA) were used in 1.0% w/w solutions with deionized water for addition to potato cakes prior to cooking. Pyrazines used as reference compounds were obtained as follows: methylpyrazine (99+%), 2,5-dimethylpyrazine (99%), 2,3-dimethylpyrazine (99%), 2,6-dimethylpyrazine (98%), ethylpyrazine (98%), 2-ethyl-3-methylpyrazine (99%), propylpyrazine (98+%), tetramethylpyrazine (98%), 2-ethyl-3,5(6)-dimethylpyrazine (99.5+%), and 2,3-diethyl-5-methylpyrazine (99%) from Fisher Scientific UK Ltd. (Loughborough, U.K.); pyrazine (99+%), trimethylpyrazine (99%), 2,3-diethylpyrazine (98%), and isobutylpyrazine (98%) from Avocado Research Chemicals (Heysham, U.K.); 2-ethyl-5(6)-methylpyrazine (98%), 2-methyl-3-propylpyrazine (97%), and 2-isobutyl-3-methylpyrazine (99%) from the Sigma-Aldrich Company Ltd. (Poole, U.K.).

Preparation and Cooking of Potato Products. Model potato cakes were prepared from drum-dried potato flake (Maris Piper:Pentland Dell 1:1) provided by McCain Foods (GB) Ltd (Scarborough, U.K.), using the method described by Elmore et al. (*9*). Samples were prepared with either unlabeled glycine or [2-^{13}C]glycine (1% w/w solution in deionized water) using potato flake and glycine solution in the ratio of 1:1.3 w/w. This gave a glycine concentration in the product of 1.3% w/w (dry weight) which was a 400-fold increase in glycine over that in the untreated potato flake (*5*). The potato flake and glycine solutions were mixed in a mechanical dough maker (Crypto-Peerless, Peerless Ltd, Halifax, U.K.). The resulting potato dough was passed through a mechanical dough roller to obtain a flattened piece of dough of about 3 mm thick. A pastry cutter (75 mm diameter) was then used to cut out the raw potato cakes from the flattened dough. To promote even baking of the potato product and to minimize bubble formation during cooking, the raw cakes were perforated with a circular dough docker. Prior to cooking, cakes were 3 mm thick and 73 mm in diameter and weighed approximately 18 g. The potato cakes were baked at 180 °C in an electric moving band impingement oven (Impinger II, Lincoln Foodservice Products Inc, Fort Wayne, IN) for 15 or 30 min. The cakes were arranged in the oven in three rows of five cakes each, with each row positioned under the upper deck hot-air jets and with the conveyor switched off. After they were baked, the cakes were allowed to cool and harden. Each batch of 15 cakes was then combined and milled to a fine powder for analysis, thereby representing the average heating conditions in the oven. The combined sample was subsequently divided into three portions, and each portion was analyzed separately to obtain triplicate analyses.

Analysis of Flavor Volatiles. Dynamic headspace concentration onto Tenax was used to isolate volatiles from the cooked potato cakes. The method described by Madruga and Mottram (*10*) was used but with the following variations. The samples (7 g) were mixed with deionized water (93 g) and were extracted at 37 °C for 1 h. Volatiles were collected on a glass-lined stainless steel trap (105 mm × 3 mm i.d.) containing 85 mg of Tenax TA (Scientific Glass Engineering Ltd, Milton Keynes, U.K.) by purging with oxygen-free nitrogen at a flow rate of 40 mL/min. Following extraction, an internal standard (100 ng of 1,2-dichlorobenzene in 1 μL methanol) was added to the trap.

Gas chromatography−mass spectrometry (GC-MS) analyses were carried out on an HP 5972 mass spectrometer, coupled to an HP 5890 gas chromatograph and a G1034C Chemstation, using a Zebron ZB-Wax column (60 m × 0.25 mm i.d., 0.25 μm film thickness; Phenomenex, U.K.). A CHIS injection port (Scientific Glass Engineering Ltd, U.K.), held at 250 °C, was used to thermally desorb the adsorbed volatiles from the Tenax trap onto the front of the column for 5 min, with a column retention gap inserted in solid CO_2. The temperature program employed was 5 min at 40 °C, a ramp of 4 °C min^{-1} to 250 °C, and then 10 min at 250 °C. The helium carrier gas flow was 1.0 mL min^{-1}. *n*-Alkanes (C_6−C_{25}) were analyzed under the same conditions to obtain linear retention indices (LRI) for the components. The mass spectrometer was operated in the electron impact mode with source temperature of 170 °C, ionizing voltage of 70 eV, and scan range from *m*/*z* 29 to 400 at 2.05 scans sec^{-1}. Compounds in samples with unlabeled glycine were identified by comparing their mass spectra and LRI values with those of authentic compounds, published LRI data, or with spectra contained in the NIST/EPA/NIH Mass Spectral Database (MS Windows version 2.0a, 2002). Compounds in samples with [2-^{13}C]glycine were identified by comparing their LRI values with those of compounds in corresponding samples with unlabeled glycine, as the presence of ^{13}C atoms in these compounds altered their mass spectra.

Determination of ^{13}C Carbon Labeling in Pyrazines and Pyrazine Mass Spectral Fragments. Hwang et al. (*11*) described a method to calculate the relative contribution of ^{14}N and ^{15}N nitrogen to pyrazine formation, in dry and aqueous model systems of glucose and glutamine-amide-^{15}N, heated at 180 °C for 1 h. This method was adapted to determine the amount of ^{13}C carbon labeling in pyrazines from samples with added [2-^{13}C]glycine. For each pyrazine, the percentages of unlabeled, singly and doubly labeled species (i.e., containing no ^{13}C atoms or one and two ^{13}C atoms, respectively, from [2-^{13}C]glycine) were calculated. No triply labeled pyrazine species were found in these samples. The amounts of ^{13}C labeling for key fragments of the pyrazine mass spectra were also calculated in a similar fashion to aid in identifying ^{13}C-label positions within the molecule. All results were corrected for the ^{13}C content of the natural isotope, as well as the possible loss of one hydrogen atom.

RESULTS AND DISCUSSION

Previous work in this laboratory using the potato model system showed that the addition of glycine to potato cakes cooked at 180 °C for 15−60 min significantly altered the alkylpyrazine distribution pattern and, to a lesser extent, the total alkylpyrazine yield (*5*). Twenty-six pyrazines were quantified as well as the precursors, 2,3-butanedione and 2,3-pentanedione. The increase in overall alkylpyrazine yield resulted from significant increases in the formation of certain key alkylpyrazines, notably those with a methyl or ethyl group in the 2- and 3-position on the pyrazine ring. These were 2,3-dimethylpyrazine, trimethylpyrazine, tetramethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, and 2,5-diethyl-3-methylpyrazine. These increases were offset by decreases in many other alkylpyrazines. In this present study using ^{13}C-labeled glycine, all alkylpyrazines which increased in the presence of glycine were found to have at least one ^{13}C atom incorporated into their structure (**Table 1**), thus confirming the participation of glycine in the formation pathways.

Alkylpyrazine Fragmentation and ^{13}C Label Distribution Patterns. The structures of the main fragments in the mass spectra of pyrazines containing ^{13}C atoms from [2-^{13}C]glycine were identified from the known fragmentation pathways for pyrazines (*12*). This allowed the position of the ^{13}C atoms within the pyrazines to be determined in many cases. The ^{13}C labeling content and label position of 2,3-butanedione and 2,3-pentanedione were also elucidated. A summary of the overall results is listed in **Table 1**. Mass spectral data obtained for pyrazines that were formed in small quantities in the control samples (particularly at 15 min) and that were further suppressed by glycine addition generally could not be used to determine the label position but were usually sufficient to determine the ^{13}C labeling

Table 1. ^{13}C-Labeling Content and Labeling Position of Alkylpyrazines and Selected Dicarbonyls in Model Potato Cakes with Added [2-^{13}C]Glycine[h] Cooked at 180 °C, Also Indicating Change in Concentration Caused by Glycine Addition

	15 min heating				30 min heating				
compound	change[a]	%UL[b]	%SL[c]	%DL[d]	change	%UL	%SL	%DL	^{13}C-labeling position
				Dicarbonyl Compounds					
2,3-butanedione	+	40.9	56.3	2.9		50.8	42.4	6.8	singly labeled at C1
2,3-pentanedione	–	76.8	20.9	2.3		57.8	34.1	8.1	singly labeled at C1 (or C2)
				Pyrazines					
methyl-	–	95.2	3.7	1.1	–	100	0.0	0.0	
2,5-dimethyl-	nc	96.0	3.7	0.3	nc	93.3	6.3	0.4	
2,6-dimethyl-	nc	56.4	42.9	0.7	nc	52.4	45.3	2.3	singly labeled at a methyl substituent
ethyl-	–	93.8	6.0	0.2	–	95.9	1.4	2.8	
2,3-dimethyl-	+	19.8	78.8	1.4	+	27.0	70.4	2.7	singly labeled at a methyl substituent
2-ethyl-6-methyl-	–	76.6	21.5	1.9	–	71.2	26.6	2.2	singly labeled at methyl substituent
2-ethyl-5-methyl-	nc	91.4	8.0	0.6	–	88.6	10.5	1.0	singly labeled at methyl substituent
trimethyl-	+	15.5	80.9	3.6	+	22.2	71.6	6.3	singly labeled at 2- or 3-methyl substituent
propyl-	–	25.8	18.6	55.7	–	45.3	38.7	16.0	at least one ^{13}C atom in propyl substituent
2,6-diethyl-[e]	–	86.6	13.4	0.0	–	90.5	8.2	1.3	
2-ethyl-3,6-dimethyl-	+	94.9	4.1	1.0	–	90.3	7.6	2.1	singly labeled at 3-methyl substituent
isobutyl-	–	nd[g]	nd	nd	–	94.1	2.4	3.5	
2,3-diethyl-	–	nd	nd	nd	–	87.9	9.8	2.3	
2,5-diethyl-[e]	nc	83.3	14.0	2.7	nc	81.2	15.3	3.6	
2-ethyl-3,5-dimethyl-	+	18.5	73.9	7.7	+	23.1	68.3	8.6	singly labeled at 3-methyl substituent
2-methyl-6-propyl-[e]	–	nd	nd	nd	–	49.8	43.5	6.7	
tetramethyl-	+	6.4	39.0	54.6	+	10.1	43.3	46.6	singly labeled at a methyl substituent; doubly labeled at 2,5- or 2,6-methyl substituents
2-methyl-3-propyl-	nc	nd	nd	nd	–	42.5	36.3	21.1	
2-methyl-6-isobutyl-[f]	nc	100	0.0	0.0	–	97.1	2.4	0.5	
2,3-diethyl-5-methyl-[e]	nc	90.1	8.7	1.2	nc	83.8	13.7	2.4	singly labeled at methyl substituent
2,6-diethyl-3-methyl-[e]	nc	90.3	8.5	1.2	–	86.5	11.6	1.9	singly labeled at methyl substituent
2,5-diethyl-3-methyl-[e]	+	33.1	45.1	21.8	+	31.6	41.8	26.7	singly labeled at methyl substituent; doubly labeled at methyl and an ethyl substituent

[a] Change in concentration compared with sample without glycine addition; +: increase, –: decrease, nc: no significant change; for details see Low et al. (*5*). [b] Percentage of unlabeled species. [c] Percentage of singly labeled species. [d] Percentage of doubly labeled species. [e] Tentative identification by comparison with literature mass spectrum and LRI (*19*). [f] Tentative identification from mass spectrum alone. [g] Not detected. [h] 1.3% w/w dry wt.

content. Examples of such pyrazines were the diethyl, propyl-, and methylpropylpyrazines. The labeling characteristics of 2-ethyl-3-methylpyrazine could not be determined because of coelution with a large quantity of trimethylpyrazine, resulting in a poor mass spectrum. However, good mass spectral data for trimethylpyrazine could be obtained because the peak was sufficiently large. It was observed that for the same glycine concentration, alkylpyrazine profiles and labeling patterns were similar irrespective of cooking time, but the overall pyrazine yields increased with cooking time. In view of the similar labeling patterns obtained with 15 and 30 min heating, average values are used in the following discussion.

Alkylpyrazine Formation Pathways. Aldol condensation type reactions between formaldehyde (the Strecker aldehyde of glycine) and other carbonyl compounds yield longer-chain α-hydroxycarbonyls containing a glycine subunit. Subsequent Strecker degradation between these longer-chain carbonyls and amino acids would produce α-amino carbonyls also containing a glycine subunit (*6*, *13*). One of the most accepted mechanisms for pyrazine formation is the condensation of two α-amino carbonyls to yield dihydropyrazines, which are subsequently oxidized to the corresponding pyrazines (*14*). This pathway has been denoted the "x + x" pathway, where "x" refers to the dicarbonyl or hydroxycarbonyl generating the aminoketone (**Figure 1**). Where one of the α-aminoketones possesses a hydroxyl group on either carbon atom adjacent to those attached to the amino or carbonyl groups, the oxidation step is not necessary. A dehydration step involving loss of this hydroxyl group from the dihydropyrazine would yield the pyrazine (*15*). If glycine participates in the x + x pathway, glycine subunits must be incorporated prior to the dihydropyrazine stage, that is, they must be introduced through the α-dicarbonyl or α-hydroxycarbonyl precursors. It is also possible for more than one glycine subunit to be incorporated into the pyrazine end product.

Glycine may also be involved in pyrazine formation through an alternative pathway, referred to as the "x + x + y" pathway (**Figure 1**). Here, "y" refers to the aldehyde whose carbon skeleton is incorporated as an additional substituent on the pyrazine ring at the dihydropyrazine stage (*16*, *17*). Strecker degradation of glycine leads to its Strecker aldehyde, formaldehyde, which may react directly with dihydropyrazine intermediates to give alkylpyrazines with an additional methyl substituent derived from glycine. However, only one substituent may be introduced into the pyrazine end product with the x + x + y pathway. Amrani-Hemaimi et al. (*16*) suggested that this mechanism is responsible for the incorporation of [3-^{13}C]alanine carbon atoms as ethyl substituents in pyrazines via interaction of acetaldehyde (the Strecker aldehyde of alanine) with dihydropyrazines. Their experiments involved [3-^{13}C]alanine and glucose/fructose model systems heated at 180 °C in coconut oil. However, Yaylayan and Keyhani (*6*) highlighted that some doubly labeled ethyl-substituted pyrazines were also observed. This suggests that another mechanism, probably x + x, occurred in parallel.

Unsubstituted Pyrazine and Pyrazines with One Substituent. Pyrazine and methyl-, ethyl-, propyl-, and isobutylpyrazine were all found to be unlabeled, that is, they contained no glycine subunits, except for propylpyrazine (**Table 1**). These pyrazines had been found to decrease in almost equal proportions (approximately 70%) with glycine addition (*5*). The single and double labeling observed in propylpyrazine suggests that the

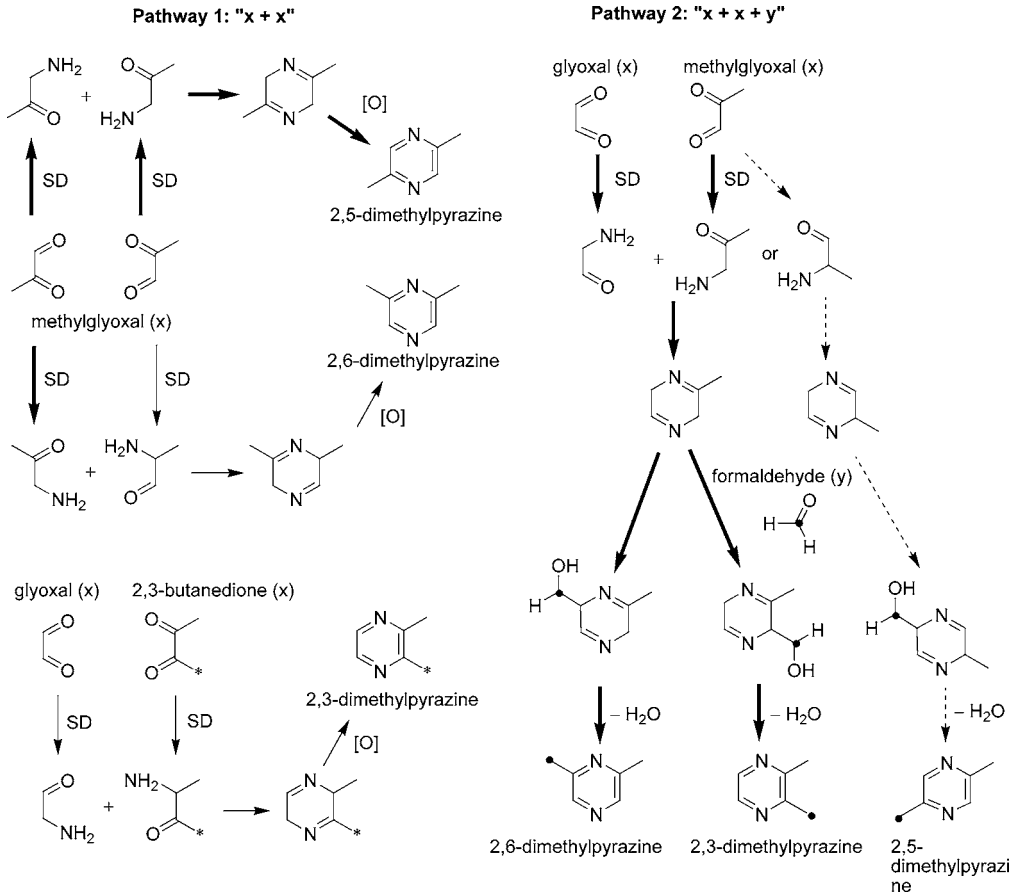

Figure 1. Two possible mechanisms for dimethylpyrazine formation, where x denotes α-dicarbonyl precursors and y denotes aldehydes that may react with the dihydropyrazine intermediate. Incorporation of ^{13}C atoms from [2-^{13}C]glycine via 2,3-butanedione is represented by ∗ and via formaldehyde by ●. Arrows in bold indicate preferred routes, and dashed arrows indicate a possible pathway which was not observed in practice. SD: Strecker degradation.

propyl substituent may have been formed from smaller carbon fragments, including those derived from glycine. That these pyrazines decreased in equal proportions suggests that they have a common precursor whose formation may have been limited by competing reactions involving glycine. An example of such a potential precursor would be glyoxal in the x + x pathway or the unsubstituted dihydropyrazine intermediate in the "x + x + y" pathway.

Dimethylpyrazines. While **Table 1** shows that 2,5-dimethylpyrazine was largely unlabeled, 2,6- and 2,3-dimethylpyrazine were singly labeled, with approximately 45% and 75% ^{13}C label incorporation, respectively. However, it had been shown previously that only the yield of 2,3-dimethylpyrazine was significantly affected by the addition of glycine, increasing by more than 2-fold (*5*). 2,5-Dimethylpyrazine was formed in the largest quantity, with or without glycine addition, followed by 2,6- and 2,3-dimethylpyrazine. The difference in yields between the isomers can be explained to a large extent by the x + x pathway (**Figure 1**). With this pathway, formation of 2,5- and 2,6-dimethylpyrazine would require the same C_3 precursors, either methylglyoxal or the corresponding hydroxycarbonyl, whereas 2,3-dimethylpyrazine would require C_2 and C_4 precursors, for example, glyoxal and 2,3-butanedione. As C_4 fragments are less abundant than C_2 and C_3 fragments (*18*), the yield of 2,3-dimethylpyrazine is therefore smaller than its isomers. At the same time, the 2,5-configuration is favored over the 2,6-configuration in the x + x pathway because amination of α-ketoaldehydes proceeds preferably at the aldehydic site, as aldehydes are generally more reactive than ketones, and this applies also to the Strecker degradation (*15*, *19*).

Since formation of the 2,5- and 2,6-isomers by the x + x pathway involves the same precursors, the presence of labeling in the 2,6- and not in the 2,5-isomer suggests that 2,6-dimethylpyrazine is formed by at least one additional route by which the ^{13}C label may be incorporated. The x + x + y pathway is one such route (*16*), with ^{13}C labeled formaldehyde (from labeled glycine) providing one of the methyl groups (**Figure 1**). If it is assumed, because of the large excess of labeled glycine, that all glycine molecules were ^{13}C labeled, the potential contribution of the x + x + y pathway to 2,6-dimethylpyrazine formation in this model system may have been as much as 45%, with the x + x pathway accounting for up to 55%. The increase of 2,3-dimethylpyrazine with glycine has been explained by the observed increase in 2,3-butanedione (*5*), which would promote the formation of this pyrazine by the x + x route (**Figure 1**). However, **Table 1** shows that 2,3-butanedione was only about 50% singly labeled, so labeling in excess of 50% in 2,3-dimethylpyrazine must be accounted for by the existence of an additional formation pathway, again probably the x + x + y pathway. The potential contribution of this latter pathway to 2,3-dimethylpyrazine formation was determined to be approximately 50% for 75% of this pyrazine to be singly labeled. This estimate assumes that 2,3-butanedione was not limiting.

Therefore, it appears that the x + x route, while an important pathway for dimethylpyrazine formation, is not the sole formation pathway for 2,6- and 2,3-dimethylpyrazine, although it is

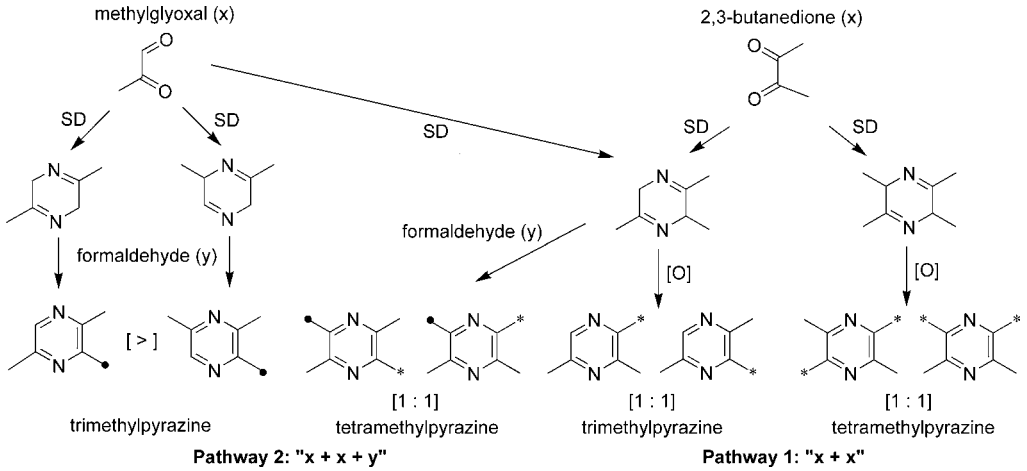

Figure 2. Two possible mechanisms for tri- and tetramethylpyrazine formation. Incorporation of ^{13}C atoms from [2-^{13}C]glycine via 2,3-butanedione is represented by ∗ and via formaldehyde by ●. Figures in square brackets indicate expected proportions. SD: Strecker degradation.

possibly the only route for 2,5-dimethylpyrazine production. 2,5-Dimethylpyrazine is typically the major pyrazine component in most model systems where pyrazines are present and does not seem to depend on the specific amino acid (*7*). This suggests that its precursors come from sugar fragmentation only and are present in abundance. On the other hand, 2,6- and 2,3-dimethylpyrazine may be expected to be more sensitive to the type of amino acid present because of the x + x + y route, which allows incorporation of part of the amino acid skeleton. On the basis of our estimate that 45−50% of 2,6- and 2,3-dimethylpyrazine was formed by the x + x + y route with glycine, the yields of these two pyrazines from this latter pathway are very similar. This implies that the addition of formaldehyde to 2-methyl-3,6-dihydropyrazine via the x + x + y route occurs at carbons 3 or 6 with equal preference. **Figure 1** also illustrates a possible route for the formation of labeled 2,5-dimethylpyrazine by the x + x + y pathway, but formation of 2,5-dimethylpyrazine via this route was not observed in practice. The significant increase in 2,3-dimethylpyrazine yield with glycine probably arises from the ability of this amino acid to promote its formation by both suggested pathways.

Other 2,3-Dimethyl Substituted Pyrazines. Like 2,3-dimethylpyrazine, the quantities of tri- and tetramethylpyrazine more than doubled with glycine addition (*5*). The increase in 2,3-butanedione production, and its involvement in the formation of these pyrazines by the x + x pathway, would be able to account for these increases, particularly for tetramethylpyrazine, which would require two molecules of 2,3-butanedione for its formation (**Figure 2**). That the largest relative increase was observed for tetramethylpyrazine and that it was found to contain up to two glycine subunits (with 2,3-butanedione having been shown to contain up to one glycine subunit) strongly support this hypothesis (**Table 1**).

However, as with 2,3-dimethylpyrazine, **Table 1** shows that a higher proportion of these pyrazines was labeled than would be explained by the x + x pathway alone, again suggesting that their formation involves at least one other pathway (x + x + y). In the case of trimethylpyrazine, about 75% was found to be singly labeled, so that the potential contribution of the x + x + y pathway would also be about 50%. For tetramethylpyrazine, the expected ^{13}C label distribution arising from the x + x pathway, on the basis of the proportion of ^{13}C labeled 2,3-butanedione, would be approximately in the ratio of 1:2:1 for the unlabeled, singly labeled, and doubly labeled species, respectively. Therefore, it would be expected that tetramethylpyrazine is 50% singly and 25% doubly labeled; however, the actual distribution was found to be approximately 40% singly and 50% doubly labeled, showing a higher percentage of the doubly labeled species, which would result from the participation of the x + x + y pathway. Therefore, this suggests that glycine is able to promote the formation of tri- and tetramethylpyrazine via both the x + x and x + x + y routes (**Figure 2**), resulting in large relative increases in both.

With the x + x pathway, the ^{13}C label position for singly labeled trimethylpyrazine would be expected to occur equally at the 2- or 3-methyl substituent, leading to equal proportions of each (**Figure 2**). With the x + x + y pathway, addition of a methyl substituent from glycine to either 2,5- or 2,6-dimethyl-3,6-dihydropyrazine would yield singly labeled trimethylpyrazine with the label at the 3- or 2-methyl substituent, respectively.

Ethylmethyl, Diethyl, Methylpropyl, and Methylisobutylpyrazines. In samples without added glycine, all the ethylmethylpyrazine isomers were formed in similar quantities, with 2-ethyl-6-methyl- being the most abundant, followed by 2-ethyl-5-methyl- and 2-ethyl-3-methylpyrazine (*5*). The higher yield of 2-ethyl-6-methylpyrazine compared to its 2-ethyl-5-methyl-isomer, and the relatively high yield of 2-ethyl-3-methylpyrazine, suggest that the x + x + y pathway plays a slightly larger role than the x + x pathway in the formation of the ethylmethylpyrazines. This is because the x + x pathway would be expected to produce more 2-ethyl-5-methylpyrazine than 2-ethyl-6-methylpyrazine as a result of amination occurring preferably at the aldehydic site of the α-ketoaldehdye precursors, while the x + x + y pathway would promote the formation of the 2-ethyl-6-methyl- and 2-ethyl-3-methyl isomers, on the basis of our observations with the dimethylpyrazines.

The presence of two different substituents gives two possibilities for the x + x + y pathway: the interaction of formaldehyde (derived from the Strecker degradation of glycine) with 2-ethyl-dihydropyrazines to form the ethylmethylpyrazines or the interaction of acetaldehyde (which may be derived from Strecker degradation of alanine) with 2-methyldihydropyrazines to form the ethylmethylpyrazines (**Figure 3**). Both formaldehyde and acetaldehyde may also be derived from sugar fragmentation but Strecker degradation would provide a ready source of these aldehydes. Since the ethyl-methylpyrazines were observed to decrease with added glycine (**Table 1**), this suggests that the x + x + y pathway involving acetaldehyde/alanine may be more important. The results obtained by Amrani-Hemaimi et al. (*16*) support this finding as the ethyl-methylpyrazines in [3-^{13}C]-alanine-hexose model systems were found to be up to 70% singly labeled, while the ethylmethylpyrazines in [2-^{13}C]glycine-

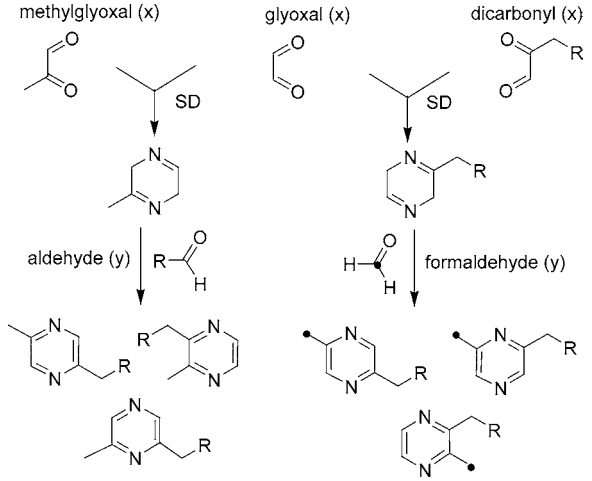

Figure 3. Possible routes for the formation of alkylpyrazines with two different substituents by the x + x + y pathway. Incorporation of ^{13}C atoms from [2-^{13}C]glycine via formaldehyde is represented by ●. Arrows in bold indicate preferred routes. SD: Strecker degradation. R = CH_3 for ethylmethylpyrazines, CH_2CH_3 for methylpropylpyrazines, and $CH(CH_3)$-CH_3 for methylisobutylpyrazines.

hexose model systems were largely unlabeled. Hence, the addition of glycine may be expected to limit the production of the ethylmethylpyrazines by competing with alanine in Strecker degradation, therefore reducing the amount of acetaldehyde formed. As mentioned, our earlier observations with the dimethylpyrazines also suggest that the addition of the ethyl group to carbon 6 would be preferred to carbon 5 in the x + x + y pathway with acetaldehyde, and this can be seen in the formation of more 2-ethyl-6-methylpyrazine than 2-ethyl-5-methylpyrazine in the control samples. On a DB-Wax or equivalent column, 2-ethyl-6-methylpyrazine would be expected to elute before the 2-ethyl-5-methyl isomer (*7*, *20*−*22*). In their paper, Amrani-Hamaimi et al. (*16*) assigned these two isomers in the opposite order, that is, they suggested 2-ethyl-6-methylpyrazine eluted after 2-ethyl-5-methylpyrazine. If taken in the generally accepted order, their results support our finding that 2-ethyl-6-methylpyrazine is formed in greater quantities by the x + x + y pathway with alanine/acetaldehyde than 2-ethyl-5-methylpyrazine.

Although the x + x + y route with alanine is more important, it can be seen from **Table 1** that the x + x + y route with glycine also plays a role in ethyl-methylpyrazine formation. Here, 25% of 2-ethyl-6-methylpyrazine and 9% of 2-ethyl-5-methylpyrazine formed were found to be singly labeled at the methyl group in the potato model system with added [2-^{13}C]-glycine. As before, it can be observed that the addition of the methyl group to carbon 6 was preferred to carbon 5, although a large excess of glycine only contributed to the formation of a small proportion of the ethyl-methylpyrazines.

Very similar observations to the ethylmethylpyrazines may be made for the diethyl-, methylpropyl-, and methylisobutylpyrazines. As with the ethylmethylpyrazines, the significant reduction in the levels of these pyrazines with glycine (*5*), and the likelihood that the 2,6-isomer predominates over the 2,5-isomer, suggests that the formation of these pyrazines involves the x + x + y route to a greater extent than the x + x route. More specifically, their formation involves the x + x + y pathway with y representing aldehydes other than formaldehyde (from Strecker degradation of glycine) (**Figure 3**). For the diethylpyrazines, this would be acetaldehyde (from alanine), and for the methyl-isobutylpyrazines, this would be methylpropanal (from valine). This is supported by the observation that both these classes of pyrazine were found to contain almost no ^{13}C label from [2-^{13}C]glycine (**Table 1**). On the other hand, the methylpropylpyrazines, in which the propyl substituent cannot be derived from a Strecker aldehyde, were found to contain some ^{13}C labeling. The pattern and extent of the labeling closely resembled that of propylpyrazine, suggesting that the labeling was located on the propyl group rather than the methyl group. It is possible that the interaction of glycine or formaldehyde with other small carbonyl fragments may have given rise to ^{13}C labeled propanal, for example, the aldol condensation of labeled formaldehyde with acetaldehyde, followed by dehydration and reduction of the resulting double bond. The propanal thus formed would then react with methyl-dihydropyrazines to give ^{13}C labeled methyl-propylpyrazines. However, competing reactions involving formaldehyde/glycine probably limited the extent of such reactions, resulting in a net decrease in methyl-propylpyrazine yields.

Ethyldimethylpyrazines. It was shown previously that addition of glycine promoted 2-ethyl-3,5-dimethylpyrazine formation significantly but had no clear effect on 2-ethyl-3,6-dimethylpyrazine, while the third isomer, 2-ethyl-5,6-dimethylpyrazine, was not detected at all (*5*). Although a 50−60% decrease in 2,3-pentanedione (one of the dicarbonyl precursors involved in this pathway) was observed with glycine addition, this did not hinder the formation of these pyrazines, particularly 2-ethyl-3,5-dimethylpyrazine, which instead was found to increase markedly with glycine. This indicates that formation of these pyrazines probably does not rely heavily on the x + x pathway and that it is likely that the x + x + y pathway plays a more important role. Again, this may involve either the addition of a methyl substituent from glycine to ethyl-methyl-dihydropyrazine or the addition of an ethyl substituent from alanine to dimethyl-dihydropyrazine (**Figure 4**). For dihydropyrazines with two alkyl substituents, the 2,5-configuration would be expected to be predominant, followed by the 2,6- and the 2,3-configuration, on the basis of our prior observations with the dimethylpyrazines. This suggests that the x + x + y pathway involving glycine would yield more 2-ethyl-3,5-dimethylpyrazine than 2-ethyl-3,6-dimethylpyrazine because of the greater availability of 2-ethyl-5-methyl-3,6-dihydropyrazine. This would explain the pronounced increase in 2-ethyl-3,5-dimethylpyrazine observed with glycine addition. This also suggests that the methyl substituent from glycine adds preferably to carbon 3 on the 2,5- and 2,6-substituted dihydropyrazines because 2-ethyl-5,6-dimethylpyrazine was not formed in significant quantities. On the other hand, the x + x + y pathway with alanine would, by the same argument, yield more 2-ethyl-3,6-dimethylpyrazine than 2-ethyl-3,5-dimethylpyrazine because of the greater availability of 2,5-dimethyl-3,6-dihydropyrazine. It was previously observed that in the control samples, about 3−5 times more 2-ethyl-3,6-dimethylpyrazine was formed than 2-ethyl-3,5-dimethylpyrazine, suggesting that without glycine addition, the pathway involving alanine is more important. This is not surprising because the alanine concentration in the potato flake used was measured to be about 5.5 times more than glycine. Therefore, it appears that both x + x + y routes with glycine and alanine are important in ethyl-dimethylpyrazine formation, in contrast to ethyl-methylpyrazine formation, where the x + x + y route with alanine appears to play a larger role.

The observed ^{13}C-label distribution in 2-ethyl-3,5-dimethylpyrazine agrees with the findings above. Approximately 70% of 2-ethyl-3,5-dimethylpyrazine was found to be singly labeled at the methyl group at carbon 3. At the same time, 2-ethyl-3,6-

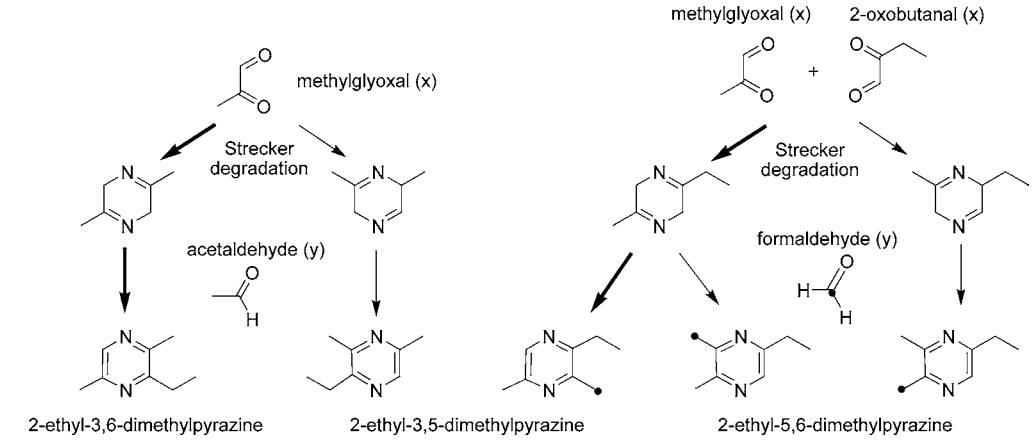

Figure 4. Possible routes for the formation of ethyldimethylpyrazines by the x + x + y pathway. Incorporation of ^{13}C atoms from [2-^{13}C]glycine via formaldehyde is represented by ●. Arrows in bold indicate preferred pathway. SD: Strecker degradation.

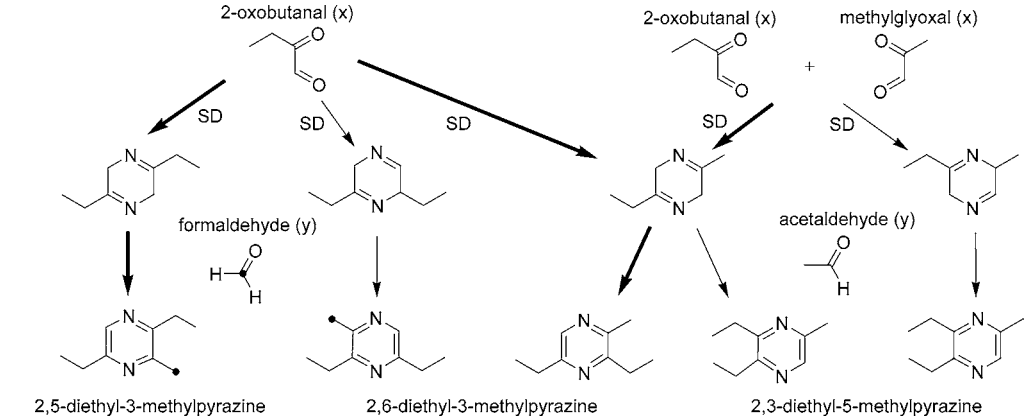

Figure 5. Possible routes for the formation of diethylmethylpyrazines by the x + x + y pathway. Incorporation of ^{13}C atoms from [2-^{13}C]glycine via formaldehyde is represented by ●. Arrows in bold indicate preferred pathway. SD: Strecker degradation.

dimethylpyrazine was largely unlabeled, although a small percentage (8%) of the 30 min sample was singly labeled, with some evidence of the label being on the methyl group at carbon 3. This suggests that 2-ethyl-3,5-dimethylpyrazine may be mostly formed from the x + x + y pathway with glycine, while 2-ethyl-3,6-dimethylpyrazine may arise mainly from the x + x + y pathway with alanine in the potato model system.

Diethylmethylpyrazines. The addition of glycine had been found to increase the yield of 2,5-diethyl-3-methylpyrazine. It had a smaller effect on the other two diethyl-methylpyrazine isomers and appeared to limit their production slightly. As with the ethyl-dimethylpyrazines, the involvement of the x + x formation pathway is probably minimal, since a decrease in 2,3-pentanedione (a key dicarbonyl precursor) did not curtail their formation significantly (*5*). In this case, the x + x + y pathway with glycine would involve the reaction of formaldehyde with diethyl-dihydropyrazine intermediates (**Figure 5**). This pathway would tend to promote the formation of 2,5-diethyl-3-methylpyrazine over its isomers because 2,5-diethyl-3,6-dihydropyrazine may be expected to be formed in larger quantities than 2,6- and 2,3-diethyl-3,6-dihydropyrazines. Therefore, the observed increase in 2,5-diethyl-3-methylpyrazine may be attributed to this formation pathway. For the x + x + y pathway with alanine, diethyl-methylpyrazine formation would involve the interaction of acetaldehyde with ethyl-methyl-dihydropyrazine. As before, it is likely that 2-ethyl-5-methyl-dihydropyrazine would be the main reactant with acetaldehyde, giving rise to either 2,3-diethyl-5-methylpyrazine, if the substituent adds to the dihydropyrazine at carbon 3, or 2,6-diethyl-5-methylpyrazine, if addition is at carbon 6. The larger quantity of 2,6-diethyl-5-methylpyrazine in the control samples (where alanine is present at a higher concentration than glycine) suggested that addition of the ethyl substituent to 2-ethyl-5-methyl-dihydropyrazine occurred preferably at carbon 6.

Much of the mass spectral evidence indicates that the diethyl-methylpyrazines (in samples with [2-^{13}C]glycine) displayed the expected ^{13}C labeling at the methyl groups. Both 2,3-diethyl-5-methylpyrazine and 2,6-diethyl-3-methylpyrazine were approximately 10% ^{13}C labeled at the methyl substituents, while 2,5-diethyl-3-methylpyrazine was 25% ^{13}C labeled at the methyl substituent in the doubly labeled species. However, with 2,5-diethyl-3-methylpyrazine, additional ^{13}C labeling at the terminal methyl group of one of the ethyl substituents suggests that there perhaps is another route other than the x + x + y by which ^{13}C labeled glycine atoms may be incorporated into this pyrazine.

Sensitivity of Alkylpyrazine Formation to Amino Acids. The preceding discussion illustrates that there is more than one chemical pathway involved in alkylpyrazine formation in complex Maillard systems. Pyrazines with a larger number and variety of alkyl substituents would have a larger number of potential formation pathways. Which route predominates depends partly on the availability of necessary precursors. The x + x route appears to be more important for pyrazines with fewer or shorter-chain alkyl substituents, while the x + x + y pathway plays a larger role in the formation of more highly substituted alkylpyrazines, particularly those with higher molecular weight or branched-chain substituents. These two pathways result not only in differences between the yields of structurally dissimilar pyrazines but also in a difference in yields between pyrazine isomers.

With the x + x pathway, sensitivity of alkylpyrazine formation to amino acid composition arises from differences in their reactivity in promoting sugar fragmentation in the Maillard reaction, which would increase alkylpyrazine formation by providing the necessary dicarbonyl and hydroxycarbonyl precursors (*8*). At the same time, some amino acids may enhance the formation of certain dicarbonyl precursors. For example, glycine has been shown to promote 2,3-butanedione formation, and alanine, 2,3-pentanedione formation, through incorporation of an amino acid subunit, in [2-^{13}C]glycine-glucose and [3-^{13}C]-alanine-glucose model systems, respectively (*6*). While these two factors also influence the x + x + y pathway, there may be additional sensitivity to α-amino acids that is able to provide an alkyl substituent, via their corresponding Strecker aldehydes, to dihydropyrazine intermediates. Such amino acids would include glycine, alanine, valine, leucine, and isoleucine. A significant increase in the concentration of one of these amino acids relative to the others may enhance the yield of those pyrazines that involve this amino acid in the x + x + y pathway at the expense of other pyrazines involving different x + x + y formation pathways. This stems from the competition between these amino acids for Strecker reagents in Strecker degradation to form the reactive aldehydes and also from competition between the Strecker aldehydes for dihydropyrazine intermediates.

Therefore, the increased sensitivity of the x + x + y route to amino acids implies that those alkylpyrazines formed mainly by this route may be more amino acid specific, for example, pyrazines containing isobutyl or isoamyl substituents. Alkylpyrazines, for example, the dimethylpyrazines, whose formation involves the x + x pathway to a greater extent, may be expected to be less amino acid specific and hence present in fairly large quantities regardless of amino acid type.

Understanding the sensitivity of pyrazine formation to amino acids provides a tool for predicting, to a certain extent, the change in alkylpyrazine profile which may be expected when an amino acid is used for acrylamide mitigation in foods. This may be used, in combination with knowledge of the odor threshold values of the pyrazines, to estimate if addition of the amino acid would have a significant effect on the flavor of the final product.

The trends in alkylpyrazine labeling patterns, which Amrani-Hemaimi et al. (*16*) observed with their simple model systems of fructose/glucose and alanine/glycine, support the results obtained in this study with the complex potato model system. However, additional information on competing alkylpyrazine pathways, and amino acid sensitivity, has been obtained with the potato model system, because of the greater diversity of precursors and alkylpyrazines present. It can be seen, therefore, that labeling studies employing real food matrixes have much potential in providing further insight into the Maillard reaction and flavor formation pathways in heated foods.

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